

Light-induced switch in barley *psbD-psbC* promoter utilization: a novel mechanism regulating chloroplast gene expression

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The synthesis of reaction center protein D₂ and mRNAs which encode this protein are differentially maintained at high levels in mature barley chloroplasts. To understand the differential maintenance of *psbD* mRNA abundance, we have studied the transcription and the RNAs produced from the *psbD-psbC* operon in plastids of light and dark-grown barley seedlings. Ten *psbD-psbC* RNAs synthesized in dark-grown barley share four different 5'-ends, two of which arise by transcription initiation, and one of which is generated by 5'-processing of longer *psbD-psbC* transcripts. Illumination of dark-grown barley causes the decline of these ten transcripts, and the accumulation of two different *psbD-psbC* RNAs. Capping assays, *in vitro* transcription and RNA processing experiments and treatment of plants with tagetitoxin (a selective inhibitor of chloroplast transcription), indicate that the light-induced transcripts arise by transcription initiation. Run-on transcription and RNA quantitation experiments provide evidence that both light-induced transcription and RNA stability play roles in the accumulation of the light-induced RNAs. These data document a novel mechanism for regulating plastid gene expression involving a light-induced switch in *psbD-psbC* promoter utilization.

Key words: chloroplast/gene/light/*psbD*/regulation

Introduction

Chloroplast biogenesis in higher plants can be divided into two overlapping phases; a build-up phase and a maintenance phase (review, Mullet, 1988). Early in the build-up phase, transcription and translation activity increase within non-photosynthetic proplastids and this is followed by the accumulation of the photosynthetic apparatus (Robertson and Laetsch, 1974; Baumgartner *et al.*, 1989). In barley, the initial activation of plastid gene expression and the accumulation of most chloroplast proteins is light independent (Klein and Mullet, 1987, 1990). However, chlorophyll and chlorophyll-apoprotein accumulation requires light (Apel, 1979; Klein *et al.*, 1988). Therefore, mature photosynthetically active chloroplasts are only formed when plants are illuminated. Once the population of chloroplasts has matured, overall rates of plastid transcription and translation decline (Mullet and Klein, 1987). This marks the transition of chloroplasts into a phase where the photosynthetic apparatus

is no longer accumulating but must be maintained to ensure continued photosynthetic activity.

The synthesis of two photosystem II reaction center proteins, D1 and D2, is differentially maintained in mature barley chloroplasts (Gamble *et al.*, 1988). The need for continued synthesis of D1 and D2 in chloroplasts may be explained by the relative instability of these proteins in illuminated plants (Mattoo *et al.*, 1984; Ohad *et al.*, 1985; Schuster *et al.*, 1988). It has been proposed that these photosystem II reaction center proteins are damaged as a consequence of photosystem II photochemistry (review Mattoo *et al.*, 1989) and in particular by blue light which may preferentially excite quinones associated with D1 and D2 (Greenberg *et al.*, 1989). This necessitates their selective turnover and resynthesis in order to maintain photosystem II activity.

The maintenance of high rates of D1 and D2 synthesis in mature barley chloroplasts is paralleled by retention of high levels of RNAs which encode these proteins (Klein and Mullet, 1986; Gamble *et al.*, 1988). In contrast, the abundance of most other plastid mRNAs declines as chloroplast populations mature (Gamble *et al.*, 1988; Mullet and Klein, 1987). D1 mRNA levels remain high in mature barley chloroplasts in part because transcription of *psbA*, the gene encoding D1, is differentially enhanced in illuminated plants (Klein and Mullet, 1990). In addition, *psbA* mRNA exhibits higher stability than many other plastid mRNAs (Mullet and Klein, 1987). *PsbA* transcription is driven by –10 and –35 promoter elements similar to other plastid and bacterial genes (Gruissem and Zurawski, 1985; Boyer and Mullet, 1988). However, at present, the molecular basis of light-stimulated *psbA* transcription is unknown.

D2 synthesis is also differentially maintained in mature barley chloroplast populations (Gamble *et al.*, 1988). Like D1, enhanced synthesis of D2 relative to other plastid-encoded proteins is paralleled by maintenance of elevated levels of mRNA encoding this protein in chloroplasts (Gamble *et al.*, 1988). However, unlike the simple transcription unit which encodes D1, *psbD*, which encodes D2, is located in a complex plastid operon. This operon encodes *psbK*, *psbI*, *psbD*, *psbC*, *orf62* and *trnG* (Oliver and Poulsen, 1984; Berends *et al.*, 1987; Neumann, 1988; Sexton *et al.*, 1990). Furthermore, at least 12 different RNAs are produced from this DNA region including two transcripts which accumulate in response to blue light (Sexton *et al.*, 1987; Gamble *et al.*, 1988). The accumulation of these latter transcripts may help account for the maintenance of D2 synthesis in barley chloroplasts.

In this study, we investigated whether *psbD-psbC* transcripts arise directly by transcription initiation or by processing of precursor transcripts. In addition, we have examined the contribution of transcription and transcript stability to the accumulation of the light-induced transcripts. We show that the light-induced *psbD-psbC* RNAs arise from

an unusual promoter which mediates transcription initiation from a 23 nucleotide region. We propose a novel transcriptional regulatory mechanism for a chloroplast operon, in which a light-induced switch in promoter utilization results in maintenance of *psbD-psbC* gene expression in chloroplasts of illuminated barley.

Results

The barley *psbD-psbC* operon

The barley chloroplast *psbD* gene is encoded in a single polycistronic operon consisting of six genes in the order *psbK-psbI-psbD-psbC-orf62-trnG* (Figure 1) which encode four polypeptides of photosystem II, a conserved open reading frame of 62 amino acids and a tRNA for glycine, respectively. Two *trnS* genes [*trnS* (GCU) and *trnS* (UGA)] are encoded by the opposite DNA strand of this same region (Sexton *et al.*, 1990). A complex series of overlapping RNAs arise from the operon (Figure 1; Berends *et al.*, 1987; Gamble *et al.*, 1988; Sexton *et al.*, 1990). The specific RNAs that accumulate and their abundance vary considerably during light-induced chloroplast development. Several RNAs containing *psbD* sequences accumulate in plastids of dark-grown plants. These RNAs are subdivided into four classes, each sharing a common 5'-terminus (Figure 1, RNAs designated by arrows preceded by solid circles, 1a–1d, 2a and 2b, 3a and 3b, 5a and 5b). For each of the four different 5'-ends, there are two corresponding clusters of 3'-ends (Berends *et al.*, 1987) which map near the divergent *trnS* and convergent *trnG* genes downstream of *psbC*. In addition, the 3'-ends of RNAs 1c and 1d lie just upstream of the 5'-end of RNAs 2a and 2b (Figure 1). Upon illumination of 4.5 day old dark-grown barley seedlings, two

additional transcripts which encode *psbD* accumulate in developing plastids (Gamble *et al.*, 1988). These transcripts, termed RNAs 4a and 4b (Figure 1, preceded by open circles), have been located ~150 nucleotides downstream of the 5'-end shared by RNAs 3a and 3b. Since a major structural feature distinguishing the five classes of transcripts is the location of their 5'-ends within the *psbD-psbC* operon, a prerequisite to studying the expression of the operon was to map the 5'-termini of the *psbD-psbC* mRNAs.

Precise mapping of 5'-ends of barley *psbD-psbC* transcripts

To map precisely the 5'-ends of the five different classes of *psbD-psbC* mRNAs illustrated in Figure 1, primer extension analysis experiments were performed on plastid RNAs from both dark and light-grown plants. The results of these assays are shown in Figure 2 along with DNA sequence generated by primer extension of the corresponding chloroplast DNA. In panel I of Figure 2, the major primer extension signal which corresponds to the 5'-end of mRNAs 1a–1d, mapped to a G residue in position 4418 of the DNA sequence, 171 bp upstream of *psbK* (Sexton *et al.*, 1990). RNAs 1a and 1b extend downstream from *psbK* through *psbD* and *psbC*, such that the first in-frame ATG codon of *psbD* is preceded by 2.07 kb of 5'-leader sequence. In panel II of Figure 2, the 5'-ends of transcripts 3a and 3b were mapped at two consecutive A residues in positions 5771 and 5772 (Sexton *et al.*, 1990), which are 712–713 nucleotides (nt) upstream of the first ATG codon of *psbD*. In panel IV of Figure 2, the major primer extension signal belonging to the 5'-end of transcripts 5a and 5b corresponds to an A residue in position 703 of DNA sequence published by Efimov *et al.* (1988). This 5'-end is located within *psbD*, 196 nt upstream of the first in-frame ATG codon of *psbC*. The 5'-end of transcripts 2a and 2b was also studied by primer extension (data not shown) and was mapped 970–978 nt upstream of *psbD* (positions 5506–5514 in Sexton *et al.*, 1990). The light-induced transcripts 4a and

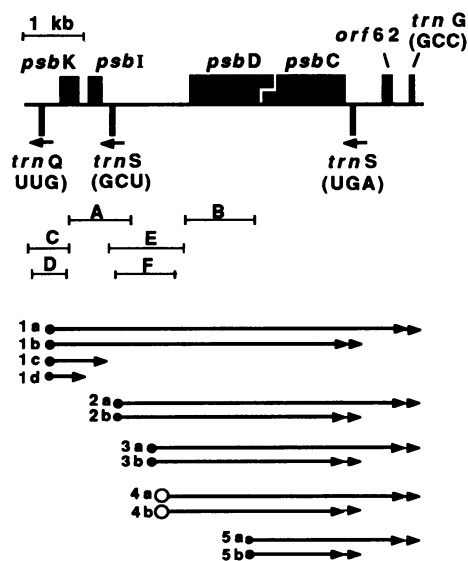


Fig. 1. Gene and transcript map of the barley chloroplast *psbD-psbC* operon. The operon encodes the loci, *psbK-psbI-psbD-psbC-trnG*, for thylakoid polypeptides of photosystem II. Genes are indicated by solid boxes. The tRNA genes below the dividing line are encoded on the opposite DNA strand as the *psbD-psbC* operon (polarity indicated by short arrow). DNA restriction fragments A–F are as referred to in the text. Mapped transcripts are designated by long arrows and labeled 1a–5b (Berends *et al.*, 1987; Gamble *et al.*, 1988; Sexton *et al.*, 1989). Solid circles indicate 5' ends of mRNAs that accumulate in etioplasts of dark-grown barley. Open circles represent 5' ends of light-induced mRNAs. Arrow heads represent 3' termini.

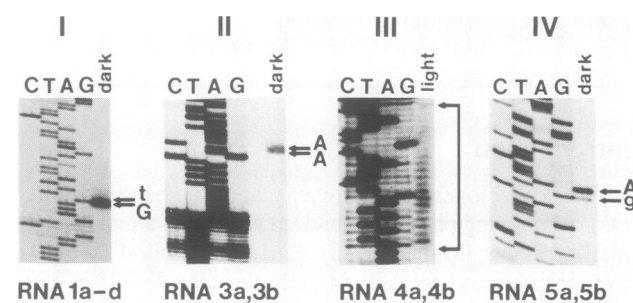


Fig. 2. Precise mapping of the 5'-ends of *psbD-psbC* transcripts. Transcripts are numbered as in Figure 1. C, T, A and G above the dideoxy sequencing reaction lanes refer to nucleotides as they appear in the published sequence (Oliver and Poulson, 1984; Neumann, 1988; Efimov *et al.*, 1988; Sexton *et al.*, 1990), so that the mRNA sequence can be read directly from the DNA sequence. Primer extension reactions on plastid RNA from 4.5 day old, dark-grown barley ('dark'; mRNAs 1a–1d, 2a, 2b, 3a, 3b, 5a, 5b) or from identical seedlings illuminated for an additional 72 h ('light'; mRNAs 4a, 4b) are shown to the right of each set of sequence ladders. Capital letters next to the primer extension lanes denote DNA nucleotides corresponding to major 5'-end signals; lower case letters point out minor primer extension signals (not all minor signals with each primer are shown here). The bracket next to panel III marks the 23 nucleotides corresponding to the heterogeneous 5'-end of light-induced transcripts 4a and 4b.

4b showed heterogeneity at their 5'-ends (Figure 2, panel III), which agrees with previous S1 nuclease and primer extension assays of this region which produced a broad signal (Gamble *et al.*, 1988). The primer extension signals corresponding to RNAs 4a and 4b span 23 nt, starting with a T residue in position 5905, and ending with an A residue in position 5927 (Sexton *et al.*, 1990). Transcripts 4a and 4b are 557–579 nt upstream of the first in-frame ATG codon of *psbD*. The 5'-ends shared by the light-induced transcripts lie 134–156 nt downstream of the 5'-end of transcripts 3a and 3b.

psbD-psbC and *rbcL* transcription and transcript stability in plastids of dark-grown and illuminated barley

When 4.5 day old dark-grown barley seedlings are illuminated for 72–108 h, RNAs 4a and 4b accumulate to become the predominant RNA species from the *psbD-psbC* operon and RNAs 1a, 1b, 2a, 2b, 3a, 3b, 5a and 5b decrease in abundance (Gamble *et al.*, 1988). This change in RNA population could result from changes in transcription, RNA processing and/or RNA stability. As a first step in determining the influence of these factors on *psbD-psbC* RNA levels, we compared light-induced changes in *psbD-psbC* transcript levels with changes in the transcription activity of these genes using lysed plastid 'run-on' transcription assays (Mullet and Klein, 1987) and RNA dot-blot hybridization experiments. For comparison, *rbcL* transcript levels and transcription activity were also examined.

The *in vivo* *psbD-psbC* and *rbcL* RNA levels were quantified on an equal plastid basis from the following four plant treatments: 4.5 day old dark-grown seedlings; 4.5 day old dark-grown seedlings, which had been illuminated for an additional 3 days; 7.5 day old dark-grown seedlings; and 7.5 day old dark-grown seedlings which had been illuminated for 4 h. The RNA probe specific for *rbcL* transcripts was derived from a linearized recombinant phagemid containing a 1.3 kb *HindIII*–*PstI* barley chloroplast DNA fragment (Zurawski *et al.*, 1984). Two different RNA probes were used to detect *psbD-psbC* RNAs. The first (probe A, Figure 1) was derived from a 1.0 kb *HindIII*–*ClaI* DNA fragment which hybridized to *psbD-psbC* RNAs 1a–1d, 2a and 2b. The second *psbD-psbC* probe (probe B, Figure 1) was derived from a 1.2 kb *EcoRI* DNA fragment which hybridized to *psbD-psbC* RNAs 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a and 5b. In addition to the plastid RNAs applied to the blots, a dilution series of *in vitro* non-radiolabeled RNA, complementary to each probe, was applied to generate a standard curve.

Plastid transcriptional activities were measured in plastids from the same four plant treatments as above and from one additional set of plants; 4.5 day old dark-grown seedlings which had been illuminated for 4 h. Plastid transcription from the *rbcL* gene and the *psbD-psbC* operon (regions covered by probes A and B, Figure 1) was quantitated by hybridization of radiolabeled plastid 'run-on' transcripts to non-radiolabeled filter-immobilized RNAs. The results presented in Table I (plastid RNA quantitation) and Table II (plastid transcription activity) show how *rbcL* and *psbD-psbC* mRNA levels and transcription vary as a function of chloroplast development and plant illumination. When 4.5 day old dark-grown seedlings were illuminated for 3 days (4.5DD+3DL, Table I) the levels of *psbD-psbC* RNAs which hybridize to region A declined by 67% and *rbcL* transcript levels by 60%.

In contrast, the *psbD-psbC* RNAs from region B declined only 35%. During this period, RNAs 4a and 4b accumulated to become the major transcripts from this DNA region (Gamble *et al.*, 1988). At the same time, transcription (Table II) from *rbcL* and from region A of *psbD-psbC* decreased similarly (by 85–86%), while transcription from region B of *psbD-psbC* decreased slightly less (by 80%). In contrast, 4 h illumination (4.5DD+4HL, Table II) caused a 49% increase in plastid transcription for *rbcL* and a 22% increase for region A. The most significant increase in transcription was observed for region B (68%).

When 4.5 day old dark-grown barley seedlings are kept in darkness for an additional 3 days (7.5DD, Table I), the levels of RNAs 1a, 1b, 1c, 1d, 2a, 2b, 3a, 3b, 5a and 5b decline slightly, and RNAs 4a and 4b remain at low levels (Gamble *et al.*, 1988). This is reflected in a 16–18% decrease in RNAs hybridizing to both probes A and B, respectively (Table I). *RbcL* RNA levels declined by 57% during this treatment. Transcription of *rbcL* and *psbD-psbC*

Table I. Quantitation of *psbD-psbC* and *rbcL* mRNA levels in plastids of dark-grown and illuminated barley

Treatment	Probe A	Probe B	
	<i>psbD-psbC</i> RNAs 1a–1d 2a, 2b	<i>psbD-psbC</i> RNAs 1a, 1b, 2a, 2b, 3a, 3b, 4a, 4b, 5a, 5b	<i>rbcL</i>
4.5DD	100%	100%	100%
4.5DD+3DL	33.0 ± 2.5%	65.2 ± 3.6%	40.0 ± 5.6%
7.5DD	84.8 ± 2.0%	82.6 ± 5.2%	41.5 ± 1.8%
7.5DD+4HL	84.8 ± 16.0%	152.0 ± 48.5%	54.9 ± 14.8%

Plastid RNA from 4.5 day old, dark-grown barley (4.5DD); 4.5 day old dark-grown barley illuminated for an additional 3 days (4.5DD+3DL); 7.5 day old dark-grown barley (7.5DD); or 7.5 day old dark-grown barley exposed to 4 h of light (7.5DD+4HL) was serially diluted and bound to a nylon membrane. After hybridization to excess single-stranded RNA probes A, B (Figure 1), or an RNA probe hybridizing to *rbcL* transcripts (see text), hybridization to each dot was quantitated by liquid scintillation counting or by using the Betascope blot analyzer (as described in Materials and methods). The hybridization level of 4.5 DD RNA was arbitrarily set at 100%. The mean of two separate experiments done in duplicate ± standard error is shown.

Table II. Quantitation of *psbD-psbC* and *rbcL* mRNA transcription in plastids of dark-grown versus illuminated barley

Treatment	<i>psbD-psbC</i> region A	<i>psbD-psbC</i> region B	<i>rbcL</i>
4.5DD	100%	100%	100%
4.5DD+4HL	122.5 ± 12.1%	168.2 ± 13.2%	149.4 ± 11.9%
4.5DD+3DL	13.9 ± 0.8%	20.6 ± 2.2%	14.4 ± 6.4%
7.5DD	52.7 ± 5.7%	43.4 ± 2.9%	15.4 ± 10.8%
7.5DD+4HL	87.6 ± 40.0%	386.9 ± 126.8%	74.4 ± 53.4%

Radioactively labeled run-on transcripts from 4.5 day old, dark-grown barley (4.5DD); 4.5 day old dark-grown barley exposed to an additional 4 h of light (4.5DD+4HL); 4.5 day old dark-grown barley illuminated for an additional 3 days (4.5DD+3DL); 7.5 day old dark-grown barley (7.5DD); or 7.5 day old dark-grown barley exposed to 4 h of light (7.5DD+4HL) were hybridized to RNA dot blots containing 1 pmol of a single stranded *rbcL* RNA probe, and 1 pmol of either probe A or B of *psbD-psbC* (see Figure 1). Hybridization was quantitated by liquid scintillation counting of the excised dots or by using the Betascope blot analyzer. Hybridization levels of A, B and *rbcL* to 4.5DD run-on transcripts were arbitrarily set at 100%. The mean of two separate experiments done in duplicate ± standard error is shown except one experiment done in duplicate for 4.5DD+4HL.

from plastids of 7.5 day old dark-grown seedlings also declined, but less than when the plants were illuminated for 3 days (compare 7.5DD with 4.5DD+3DL, Table II). When the 7.5 day old dark-grown seedlings were illuminated for 4 h (7.5DD+4HL, Table I), the level of *psbD-psbC* RNAs 1a–1d, 2a and 2b did not change, *rbcL* mRNA levels increased to a small extent and *psbD-psbC* RNAs hybridizing to probe B increased ~2-fold. Transcription from *rbcL* and both *psbD-psbC* regions assayed (7.5DD+4HL, Table II) increased with the greatest stimulation occurring in the *psbD-psbC* DNA region covered by probe B. A large standard error was observed in transcription assays of 7.5 day dark 4 h illuminated plants which we attribute to the relatively rapid increase in plastid transcription which occurs upon illumination of 7.5 day old dark-grown plants (Klein and Mullet, 1990).

The overall decline in plastid transcription activity for all DNA regions assayed in dark treated or illuminated plants during the period from 4.5 to 7.5 days exceeded decreases in RNA levels indicating that the stability of the transcripts assayed influences their *in vivo* steady-state levels. However, a significant induction of transcription by short periods of illumination (4 h), is one indication that light-induced transcription, in addition to RNA stability, plays a role in regulating the expression of this operon during plastid development. Moreover, transcription of region B (Figure 1), was stimulated by light more than region A even though region B is located 3' to region A in the *psbD-psbC* operon. These results led us to investigate the possible existence of multiple promoters within the operon and whether RNAs 4a and 4b were primary transcripts which arose by light-induced transcription.

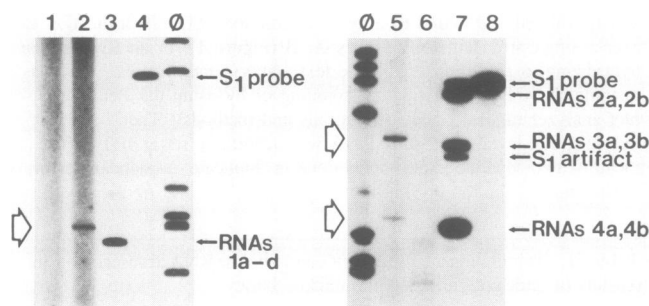


Fig. 3. Capping of *in vivo psbD-psbC* transcripts. Lanes 1–4 concern the 5'-end region of transcripts 1a–1d. Lanes 1 and 2 show the results of S1 nuclease treatment of capped plastid RNA from 4.5 day old dark-grown barley, with (lane 2) or without (lane 1) prior hybridization to an unlabeled 0.59 kb *EcoRV-HindIII* fragment (lane 4). The open arrow points to the protected RNA fragment in lane 2. Lane 3 shows the corresponding standard S1 nuclease assay on unlabeled etioplast RNA, using end-labeled *EcoRV-HindIII* fragment. The major 5'-end signal in lane 3 corresponding to transcripts 1a–1d has been marked. Lanes 5–8 concern the 5'-end regions of transcripts 2a, 2b, 3a, 3b, 4a and 4b. Lanes 5 and 6 show the results of S1 nuclease treatment of capped plastid RNA from 4.5 day old dark-grown barley, illuminated for an additional 72 h, with (lane 5) or without (lane 6) prior hybridization to an unlabeled version of the 0.87 kb *EcoRI* fragment shown in lane 8. Open arrows point to the protected RNA fragments in lane 5. Lane 7 shows the corresponding S1 nuclease assay on unlabeled plastid RNA, using the labeled probe in lane 8. The S1 artifact signal has been previously described (Sexton *et al.*, 1990). The S1 signals in lane 7 representing the 5' ends of transcripts 2a and 2b, 3a and 3b, 4a and 4b are as marked. ϕ refers to 5'-end labeled ϕ X174-*HaeIII* marker DNA.

Capping of *in vivo psbD-psbC* transcripts

To examine the possibility of transcription initiation at more than one promoter in the operon, we used capping assays to identify *psbD-psbC* primary transcripts (Moss, 1977). The results of two capping experiments, one concerning the 5'-end shared by mRNAs 1a–1d, the other concerning the 5'-ends of transcripts 2a and 2b, 3a and 3b, 4a and 4b are presented in Figure 3. In the first experiment (lanes 1–4), barley etioplast RNA protected 0.26 kb of an end-labeled *EcoRV-HindIII* DNA probe (DNA region C, Figure 1) from S1 nuclease digestion (lane 3). Unlabeled *EcoRV-HindIII* (DNA) was able to protect an RNA, radiolabeled at its 5'-ends by capping, of a similar size (lane 2). The capped RNA corresponded to the 5'-ends of RNAs 1a–1d which is evidence that they are primary transcripts. This capped, protected RNA migrated slightly slower than the end-labeled DNA (lane 3) protected by plastid RNA in S1 nuclease assays. Such mobility differences between capped, protected RNA/DNA fragments of equal size have been previously reported and are presumably due to the addition of GMP to the RNA during the capping reaction and differences in the electrophoretic migration of RNA and DNA (Boyer and Mullet, 1986).

In a second experiment (Figure 4, lanes 5–8), chloroplast RNA protected three portions of an end-labeled 0.87 kb *EcoRI* DNA fragment (fragment E in Figure 1) from S1 nuclease digestion (Figure 4, lane 7 and Gamble *et al.*, 1988). These three protected fragments delineate the 5'-ends of RNAs 2a and 2b, 3a and 3b, and light-induced RNAs 4a and 4b. A previously described band arising from spurious S1 nuclease cleavage is also noted in lane 7 (Berends *et al.*, 1987). In contrast, unlabeled 0.87 kb *EcoRI* DNA was able to protect only two RNA fragments which had been radiolabeled with [α - 32 P]GTP by guanylyltransferase (lane 5). Again, the protected, capped RNAs migrated slightly more slowly than the two corresponding DNA fragments protected by RNAs 3a and 3b or 4a and 4b (compare lanes 5 and 7). These results indicate that transcripts 3a, 3b, 4a and 4b are primary transcripts. In contrast, RNAs 2a and 2b were not capped *in vitro*, and they may arise from RNA processing. It should be noted that the intensities of the capped RNA bands in lane 5 are reversed relative to the corresponding labeled DNA bands in lane 7. This may reflect differences in capping efficiency, or that a portion of RNAs 4a and 4b are modified by RNA processing. Extensive RNA secondary structure around a 5'-end can dramatically decrease capping efficiency (Moss, 1977). Since the capping experiments provide evidence that light-induced RNAs 4a and 4b are primary transcripts, we addressed the question of whether transcription was required to observe light-induced accumulation of these RNAs.

Accumulation of RNAs 4a and 4b required light-induced transcription

The effect of pretreating seedlings with tagetitoxin, a selective inhibitor of chloroplast RNA polymerase (Mathews and Durbin, 1990), on the ability of light to induce the accumulation of RNAs 4a and 4b is shown in Figure 4. Plant nuclear RNA polymerases are much less sensitive to tagetitoxin, hence the experiments focus on the specific effects of the toxin on plastid transcription. Primer extension assays using a 135 bp *Sau96I-EcoRI* primer (Gamble *et al.*, 1988) were conducted on total RNAs isolated from plants

treated as described in Figure 4 and Materials and methods. RNAs 2a, 2b, 3a and 3b are detected in both intact and excised 4.5 day old dark-grown barley seedlings. A low level of RNAs 4a and 4b was also detected in dark-grown seedlings. Because their 5'-ends span a 23 nt region, signals for these RNAs are represented as a doublet band on 8% polyacrylamide gels. Upon transfer of the seedlings to the light for 4 h, RNAs 4a and 4b accumulate in both intact and, to a slightly diminished degree, in excised seedlings (Figure 4, lanes 5 and 6). Interestingly, most of the RNA that accumulated after 4 h illumination corresponded to the lower band of the 4a/4b doublet. Pretreatment of 4.5 day old dark-grown barley seedlings with tagetitoxin abolished the ability of light to induce the accumulation of RNAs 4a and 4b. Uptake of tagetitoxin was equally effective through the roots or directly up stems of excised seedlings. In tagetitoxin treated seedlings, primer extension signals for the RNAs present in dark grown plants (RNAs 2a, 2b, 3a and 3b) are still present after the 12 h tagetitoxin pretreatment. The abundance of RNAs 2a and 2b was decreased by this treatment while the level of RNAs 3a and 3b was unchanged. This could mean that under conditions in which plastid transcription is inhibited *in vivo*, RNAs 2a and 2b are less stable than RNAs 3a and 3b. It should be noted that the time course of transcription inhibition by tagetitoxin cannot be easily determined in these *in vivo* experiments.

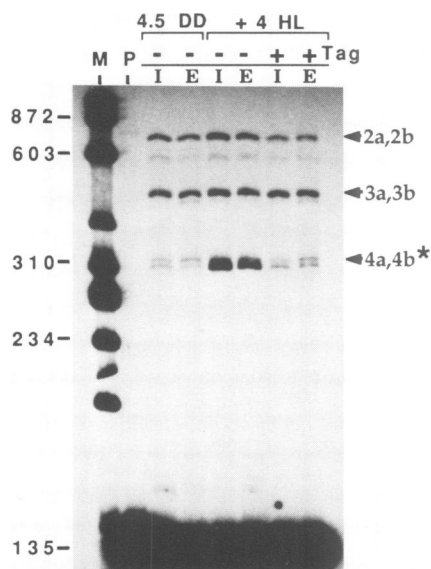


Fig. 4. Effect of tagetitoxin and light on the accumulation of *psbD-psbC* mRNAs. Primer extension assays were conducted on total RNAs from barley seedlings of six different treatments as designated by symbols at the top of each lane of the 8% polyacrylamide-urea gels used to separate the reaction products. Seedlings were either 4.5 day old, dark-grown (4.5DD) or grown with an additional 4 h of light (+4HL). The symbols + and - indicate plus or minus tagetitoxin (TAG) pretreatment. Concentrations of tagetitoxin in the pretreatment solutions were 100 μ M for intact and 180 μ M for excised seedlings. Lanes marked I and E refer to seedlings that were either intact or excised, respectively, before tagetitoxin pretreatment. Lane M refers to some of the 32 P-end labeled ϕ X174-*Hae*III restriction fragments used as size (bp) markers. Lane P refers only to the primer, a 135 bp *Sau*96I-*Eco*RI restriction fragment, used in the reactions. Arrowheads designate signals for RNAs 2a, 2b, 3a, 3b, 4a and 4b as described in Figure 1. The asterisk denotes the light-induced transcripts.

Barley *psbD-psbC* promoter activity in a pea *in vitro* transcription extract

We next tested the ability of specific regions of the *psbD-psbC* operon to promote transcription in a pea chloroplast *in vitro* transcription extract. Pea chloroplast extracts were previously shown to initiate transcription accurately on heterologous templates (Orozco *et al.*, 1985). These extracts also lack barley plastid RNAs which would complicate the analysis of transcription from barley DNA templates. Results are shown in Figure 5. The 463 bp *Bst*BI-*Spe*I DNA fragment (region D, Figure 1), which contains sequences around the 5'-end of transcripts 1a-1d was tested first. A pTZ19T plasmid vector (Chen and Orozco, 1988), with (lane 3) or without (lane 4) this barley chloroplast DNA insert, was added to pea plastid transcription extracts in the presence of unlabeled nucleotides. RNA generated in the extract was subsequently isolated, and subjected to primer extension analysis using a primer specific for RNAs synthesized from the barley chloroplast DNA insert. Primer extension analysis of plastid RNA from 4.5 day old dark-grown barley yielded

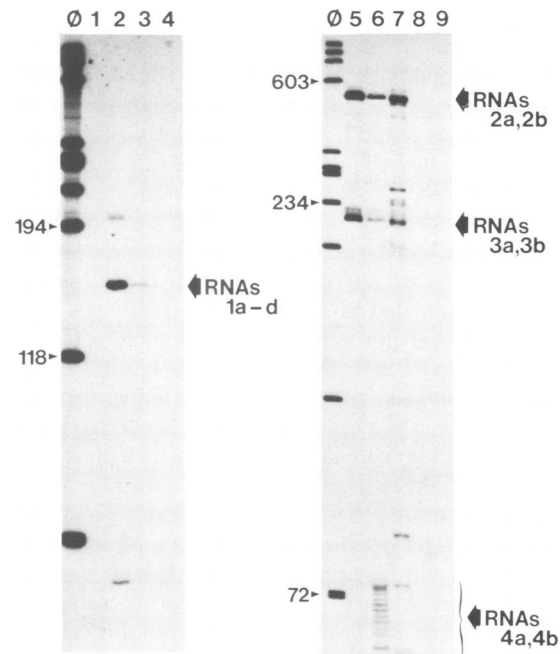


Fig. 5. Barley *psbD-psbC* promoter activity in a pea *in vitro* transcription extract. **Lanes 1-4** RNAs produced from a 463 bp *Bst*BI-*Spe*I DNA region were analyzed by primer extension assays. The primer used for these assays was run in lane 1. In lane 2, *in vivo* etioplast RNAs were analyzed. RNAs analyzed in lanes 3 and 4 were produced *in vitro* in pea extracts with (lane 3) or without (lane 4) the added 463 bp insert. The 5'-end signal of transcripts 1a-1d has been marked. **Lanes 5-9** concern the 668 bp *Bst*BI fragment which contains the 5'-ends of barley transcripts 2a, 2b, 3a, 3b, 4a and 4b. Control primer extension assays on plastid RNA from 4.5 day old dark-grown barley, or from identical seedlings illuminated for an additional 72 h are shown in lanes 5 and 6 respectively. RNAs transcribed in the pea extract with or without the added 668 bp insert were used in the primer extension assays in lanes 7 and 8 respectively. The primer used for these assays was run in lane 9. The large arrows point to the 5'-end signals of transcripts 2a and 2b, 3a and 3b, 4a and 4b.

a major 152 nt primer extension signal (lane 2, large arrow) corresponding to the 5'-end of transcripts 1a–1d. This RNA was not produced by addition of vector DNA to the pea extract (lane 4). However, when plasmid DNA containing the barley chloroplast DNA insert was transcribed in the pea extract, the 152 nt band was produced (lane 2, large arrow). This indicates that promoter activity within the 463 bp *Bst*BI–*Spe*I fragment results in transcription initiation at a site corresponding to the 5'-end of *psbD-psbC* RNAs 1a–1d.

The second barley DNA tested was a 668 bp *Bst*BI fragment (region F, Figure 1A) which extended 343 bp upstream of the 5'-end of transcripts 2a and 2b, and 169 bp downstream of the 5'-ends of light-induced transcripts 4a and 4b. A control primer extension assay, again with a barley insert specific primer, on plastid RNA from 4.5 day old dark-grown barley (lane 5) or plastid RNA from identical seedlings illuminated for an additional 72 h (lane 6) revealed the 5'-end signals of light-induced transcripts 4a and 4b (bracket, large arrow), and transcripts 2a and 2b, 3a and 3b (marked to the right of the figure by arrows). Plasmid DNA containing the 668 bp insert transcribed in pea extracts gave rise to several bands (lane 7), none of which appeared when the vector minus insert was used (lane 8). Some of the bands in lane 7 did not co-migrate with the 5'-end signals in lanes 5 and 6, possibly reflecting general differences between pea and barley. However, an RNA was produced *in vitro* with its 5'-end identical to transcripts 3a and 3b (compare lanes 5 and 6 with 7). Similarly, three bands in lane 7 co-migrated with DNA fragments protected by *in vivo* RNAs 4a and 4b. This suggests that the pea extract can direct transcription at some positions within the 668 bp *Bst*BI fragment which give rise to light-induced transcripts 4a and 4b. While this data is consistent with the presence of a promoter proximal to RNAs 4a and 4b, clearly pea transcription extracts did not generate the complete set of RNAs observed *in vivo* in barley. This may be due to general differences between pea and barley or reflect the lack of a specific factor in pea extracts which is needed to transcribe this DNA in illuminated barley plastids. Pea plastids do not show the light-induced switch in *psbD-psbC* RNA populations observed in barley (Sexton and Mullet, unpublished). The development of barley plastid transcription extracts will be needed to resolve these possibilities. An intense band in lane 7 co-migrated with signals generated by RNAs 2a and 2b. *In vitro* generation of this signal in the pea extract could represent a non-cappable transcription initiation point, or could be explained by transcription initiation further upstream, followed by post-transcriptional processing of larger precursor RNAs to produce RNA 5'-ends corresponding to RNAs 2a and 2b.

Processing of barley *psbD-psbC* RNAs in plastid extracts of pea and barley

Since some of the RNAs generated in the pea extract could be derived from processing of larger RNAs which arose by *in vitro* transcription, we tested the ability of pea and barley plastid extracts to process RNA derived from the barley *psbD-psbC* operon. The RNA substrate consisted of a gel-purified 0.87 kb transcript synthesized by T7 RNA polymerase from region E (Figure 1) which spanned the 5'-termini of RNAs 2a, 2b, 3a, 3b, 4a and 4b. For comparison, the primer extension assays of the *in vivo* RNAs

are shown in Figure 6A (lanes 1–3). The *in vivo* accumulation of RNAs 4a and 4b and the concomitant decrease of RNAs 2a, 2b, 3a and 3b in plastids of 4.5 day old dark grown

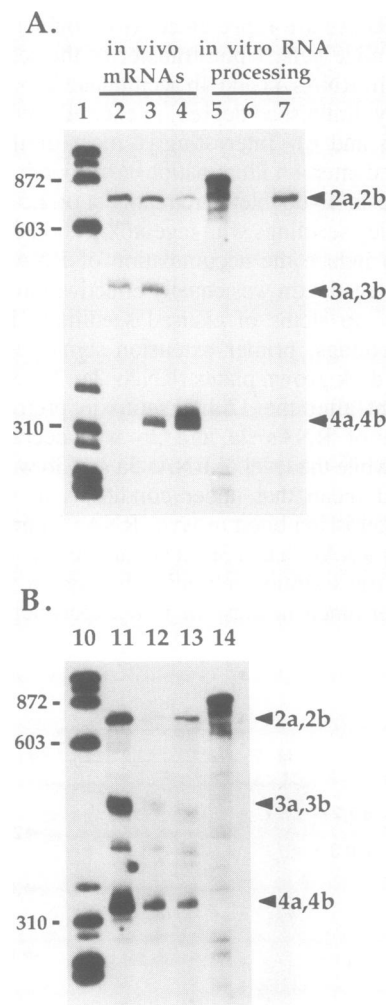


Fig. 6. *In vitro* RNA processing activities in pea and barley transcription extracts. RNAs were assayed in primer extension analysis experiments using the 135 bp *Sau*96I–*Eco*RI restriction fragment, complementary to *psbD-psbC* mRNAs, as a primer labeled at the *Eco*RI site (Gamble *et al.*, 1988). The numbers next to lanes 1 and 10 refer to the sizes (bp) of some of the end-labeled ϕ X174–*Hae*III restriction fragments used as molecular size markers. Large arrowheads refer to the 5'-end signals of transcripts described in the text and shown in Figure 1. (A) A gel-purified, synthetic 0.87 kb transcript which spans the 5'-ends of barley *psbD-psbC* RNAs 2a, 2b, 3a, 3b, 4a and 4b, was tested *in vitro* as a processing substrate in the plastid extract from pea (7 day old, 16 h light/8 h dark cycle) seedlings. RNA processing was assayed by primer extension of the 0.87 kb transcript before (lane 5) and after addition of 80 ng to the pea (lane 7) plastid extract for 60 min at 30°C. A negative control assay is shown for the RNAs in pea (lane 6) extracts. Positive control primer extension assays are shown for *in vivo* plastid RNAs from 4.5 day old, dark-grown barley without (lane 2) or with an additional 4 h (lane 3) and 72 h (lane 4) of illumination. (B) Analysis of RNA processing in plastid extracts from 4.5 day old dark-grown barley seedlings illuminated with an additional 4 h of light. Primer extension assays were conducted on the endogenous RNAs present in plastid extracts before (lane 11, extract kept on ice) and after (lane 12) incubation of the extract for 60 min at 30°C. Assays were also conducted on 80 ng of the gel-purified, synthetic 0.87 kb transcript before (lane 14) and after (lane 13) incubation of the transcript in the plastid extract for 60 min at 30°C.

seedlings are readily discernible after 4 h (lane 3) and 72 h (lane 4) of illumination. Most of the 0.87 kb *in vitro* synthesized transcript preparation gave rise to full-length primer extension signals prior to its addition to the extracts (lanes 5 and 14, Figure 6A and B). Smaller signals could have been produced by interference of the reverse transcriptase by secondary structure of antisense tRNA-Ser present in this RNA. After incubation of the 0.87 kb *in vitro* RNA in plastid extracts for 60 min, a prominent band was detected which co-migrated with the primer extension signals corresponding to *in vivo* RNAs 2a and 2b (compare lanes 2–3 with lane 7, Figure 6A). This suggests that RNA 5'-ends corresponding to the termini of RNAs 2a and 2b can be generated by RNA processing in the pea plastid extracts. Incubation of the 0.87 kb transcript in the pea extract did not, however, result in the appearance of 5'-end signals belonging to transcripts 3a and 3b, 4a and 4b (lanes 6 and 7, large arrows, Figure 6A).

The possibility that a processing activity in extracts of plastids from illuminated barley would give rise to the light-induced RNAs 4a and 4b was tested (Figure 6B). The presence of endogenous RNAs in the plastid extract was examined first. RNAs 2a, 2b, 3a, 3b, 4a and 4b are readily detectable in primer extension assays of the barley plastid extract (incubated on ice, lane 11, Figure 6B) from 4.5 day old dark-grown seedlings exposed to 4 h of light. After incubation for 60 min (lane 12, Figure 6B), the levels of RNAs 2a, 2b, 3a and 3b had decreased to trace amounts, while slightly higher relative levels of RNAs 4a and 4b remained. This may indicate that the stability of RNAs 4a and 4b is greater than the other *psbD-psbC* RNAs. Next, plastid extracts from barley seedlings exposed to 4 h illumination were assayed for their ability to process the 0.87 kb transcript. An equivalent amount of the 0.87 kb transcript as shown in lane 14 (Figure 6B) was added to the barley plastid extract, incubated for 60 min and the resulting products assayed by primer extension analysis (lane 13, Figure 6B). A primer extension signal co-migrating with the signals for RNAs 2a and 2b was detected. This result indicates that the 5'-ends of RNAs 2a and 2b can be generated by RNA processing in barley plastid extracts.

Discussion

We have studied the transcription of, and RNAs produced from, the *psbD-psbC* operon in barley chloroplasts. Special

emphasis was placed on the mechanisms regulating the differential accumulation of *psbD-psbC* RNAs during light-induced chloroplast biogenesis. The results presented here demonstrate that much of the RNA heterogeneity is due to the action of at least three promoters which drive three overlapping transcription units in the *psbD-psbC* operon. One of these promoters is differentially activated in illuminated plants. This switch in promoter utilization is novel for a plastid operon. An intriguing aspect of the light-induced promoter switch is its role in the maintenance of the expression of *psbD-psbC* during chloroplast maturation.

Occurrence of multiple promoters in the barley plastid *psbD-psbC* operon

The mRNA diversity arising from many of the chloroplast polycistronic operons has been explained by 5' and internal endonucleolytic cleavage events (Crossland *et al.*, 1984; Mullet *et al.*, 1985; Hudson *et al.*, 1987; Westhoff and Hermann, 1988; Christopher and Hallick, 1990), 3'-exonucleolytic processing reactions (Stern and Gruissem, 1989) and intermediates in splicing (Rock *et al.*, 1987; Barkan, 1989; for review see Gruissem, 1988). Here, capping, *in vitro* transcription and *in vitro* RNA processing experiments provide evidence that the 5'-ends of *psbD-psbC* RNAs 1a–1d, 3a, 3b, 4a and 4b, arise by transcription initiation, while RNAs 2a and 2b arise by RNA processing of larger RNAs 1a and 1b. Therefore, much of the *psbD-psbC* mRNA diversity can be explained by transcription initiation at multiple promoters within the operon. Recently, multiple promoters have also been observed for two divergent maize plastid polycistronic operons (Haley and Bogorad, 1990), for *psbD-psbC* in tobacco (Yao *et al.*, 1989) and pea (Woodbury *et al.*, 1989) and *atpB* in spinach (Chen *et al.*, 1990). Thus in higher plant chloroplasts, it is apparent that some polycistronic operons are comprised of multiple overlapping transcription units.

DNA sequences upstream of the 5'-end of RNAs 1a–1d (Figure 7) are similar to plastid and *Escherichia coli* –10 and –35 transcription promoter elements (Gruissem and Zurawski, 1985a,b; Rosenberg and Court, 1979; Kung and Lin, 1985; for reviews see Hanley-Bowdoin and Chua, 1987). This is not surprising because this DNA region was able to promote transcription *in vitro* and the 5'-end of RNAs 1a–1d were capped by guanylyltransferase. Sequences upstream of the 5'-ends of RNAs 2a and 2b do not contain recognizable promoter elements, these RNAs were unable

TTGaca	TATaaT		<u>E. coli</u> consensus
-35	-10	5' termini	
TCGAAAAAATGCATTCGATCATTACATGGAATTCATAGATATATAT		<u>G</u>	RNAs 1a–1d
AACGGATTAGCAATCCGCCGCTTTAGTCCACTCAGCCATCTCTCCACGTT		<u>CCAAAGCGA</u>	RNAs 2a, 2b
TCTCTTTAGCGGGCATTTCCATATAGGACTTGTATAAATATAATAAAC		<u>AA</u>	RNAs 3a, 3b
AAATTGCAAGAGAAGCATAAAGTAAGTAGACCTGACTCCTGAAATGATGCC		<u>TCTATCCGCATTCGATATATA</u>	RNAs 4a, 4b
CCCAAACTCTTGGTGTGCTTTTCCAATAAACGTTGGTACATTTCTTT		<u>A</u>	RNAs 5a, 5b

Fig. 7. DNA sequence comparison of 5'-flanking regions. The DNA nucleotides corresponding to the major 5'-ends of the *psbD-psbC* transcripts are underlined. For each of the 5'-ends, 50 nt of 5'-flanking region is shown. Sequences with homology to –35 and –10 consensus elements are boxed. Consensus bacterial –10 and –35 promoter elements are shown at the top of the figure.

to be capped and RNAs with 5'-ends corresponding to RNAs 2a and 2b could be generated by RNA cleavage in plastid extracts. The *trnS* (GCU), which is located 6–14 bp upstream from the 5'-end of RNAs 2a and 2b (Sexton *et al.*, 1990), could serve as an RNA processing site. Sequences upstream from the 5'-end of RNAs 3a and 3b exhibit poor similarity to the consensus –35 promoter element but strong similarity to the –10 promoter element (Figure 7). RNAs 3a and 3b were readily capped and the DNA region encoding these RNAs was utilized as a promoter *in vitro*. Taken together, this evidence supports the conclusion that RNAs 3a and 3b result from transcription initiation from a second promoter without additional 5'-end RNA processing. The light-induced RNAs 4a and 4b also do not appear to arise by RNA processing in plastid extracts of light grown plants, confirming the results from the capping, tetracycline inhibition and *in vitro* transcription experiments. However, putative promoter elements used for transcription of RNAs 4a and 4b do not appear to be typical for chloroplasts (Figure 7). The 5'-ends shared by RNAs 4a and 4b covered a 23 nucleotide region. Heterogeneity for transcription start site scattered over a 30 bp region has also been observed for some genes in *E.coli* (Jacquet and Reiss, 1990). Finally, RNAs 5a and 5b share 5'-ends which map within *psbD* (Sexton *et al.*, 1987). Upstream of these termini are DNA sequences which show homology to consensus promoter elements. In this study we did not test if this DNA region in barley is a promoter. However, similar DNA regions within tobacco and pea *psbD* loci were recently identified as a site of *psbC* transcription initiation (Yao *et al.*, 1989; Woodbury *et al.*, 1989).

Light-induced switch in *psbD-psbC* promoter utilization and the role of RNA stability

Light-induced transcription from a promoter that is relatively inactive in dark-grown plants was found to be necessary for the differential accumulation of RNAs 4a and 4b upon illumination of barley. However, transcription alone could not explain the differential accumulation of RNAs 4a and 4b in chloroplasts. During later stages of chloroplast maturation, transcription of RNAs 4a and 4b decreased more than the abundance of these RNAs. Therefore, the stability of RNAs 4a and 4b is also an important factor determining their levels *in vivo*. Consistent with this possibility, RNAs 4a and 4b appeared to be more resistant than RNAs 3a, 3b, 2a and 2b to the nucleases present in plastid extracts of illuminated barley plants. The 3'-inverted repeats of spinach plastid mRNAs have been shown to function in RNA stabilization and processing (Stern *et al.*, 1989). However, an obvious structural feature distinguishing the transcripts studied here is the different lengths of their 5'-ends. Their enhanced stability could be due to sequences located at or near their 5'-termini. Maize plastid polycistronic transcripts differing in their 5'-leader regions also accumulate differentially (Haley and Bogorad, 1990) and in bacterial systems, RNA stability can be affected by the nature of sequences at the 5'-end of the RNAs (Brawerman, 1989).

Rationale for the maintenance of *psbD-psbC* mRNA in chloroplast populations

During the 'build-up' phase of the chloroplast development, *psbD-psbC* RNAs 1a, 1b, 2a, 2b, 3a, 3b, 5a and 5b are

present at relatively high levels. If plants are illuminated during the build-up phase of chloroplast biogenesis, *psbD-psbC* RNAs 4a and 4b also accumulate. Once the mature population of leaf chloroplasts is formed, overall plastid transcription activity declines ~10-fold. However, in barley, mRNAs encoding D1 and D2 are maintained at high levels in mature chloroplasts in contrast to other RNAs which decline once mature chloroplast populations are formed (Mullet and Klein, 1987; Klein and Mullet, 1987; Gamble *et al.*, 1988). In this paper we show that the action of the light-induced promoter plus relatively high transcript stability combine to maintain *psbD* transcript levels in fully developed chloroplast populations. This allows continued synthesis of D2 in mature chloroplasts, ensuring a supply of D2 subunits to replace those damaged or degraded in illuminated chloroplasts.

Differential regulation of spinach plastid gene expression has been observed to occur primarily at post-transcriptional levels (Deng and Gruissem, 1987, 1988). In barley chloroplasts, however, transcription of *psbA* (Klein and Mullet, 1990) and *psbD-psbC* is differentially enhanced upon illumination of dark-grown seedlings. Therefore, transcriptional regulation may occupy a more prominent role in barley than in spinach chloroplasts. The factors involved in the differential transcription of plastid genes in light versus dark have not yet been established. In the case of *psbD-psbC* mRNAs 4a and 4b, photosensitization of a blue light photoreceptor (Gamble and Mullet, 1989) activates transcription from a *psbD-psbC* promoter which is relatively inactive in dark-grown plants. Implication of a nuclear gene product required to activate this promoter (Gamble and Mullet, 1989) and the unusual DNA sequences upstream of the transcription initiation site are reminiscent of bacterial promoters which require special sigma factors for activity (Fujita *et al.*, 1987; Carter and Moran, 1986).

Materials and methods

Plant growth

Barley (*Hordeum vulgare* L. var. Morex) and pea (*Pisum sativum*, progress No. 9) seeds were planted in vermiculite watered with half-strength Hoagland's nutrient solution. Seedlings were maintained in light-tight controlled environment chambers (at 23°C) in a light-tight room. After 4.5 or 7.5 days, barley seedlings were either harvested or transferred to a continuously illuminated chamber with a light intensity of 123 W/m²/s (fluorescent plus incandescent bulbs). Pea seedlings were transferred after 4 days to a chamber for 112 h with a 16 h light/8 h dark cycle. All manipulations of dark-grown plants were performed in complete darkness. However, when necessary, a dim green safelight was used as described by Klein and Mullet (1986).

Plastid and plastid nucleic acid isolation

Plastids were isolated on percoll gradients as previously described (Klein and Mullet, 1986). Intact plastids were quantitated (in terms of plastid number per μ l of suspension volume) by phase contrast microscopy using a hemacytometer. Total nucleic acid was isolated from intact plastids as previously described (Mullet *et al.*, 1985) and resuspended in H₂O on a per plastid basis. Total nucleic acid was treated with RQ1-DNase (Promega Biotech), phenol extracted and EtOH-precipitated to yield plastid RNA.

Preparation of transcriptionally active extracts from plastids

Intact plastids were purified as described above. The transcriptionally active 'high-salt' extract was prepared essentially according to Orozco *et al.* (1985). After the final dialysis step in 1 × DEAE buffer (50 mM Tricine-KOH pH 8, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, 0.5 mM phenylmethylsulfonylfluoride, 1 mM benzamidide, and 5% glycerol), the extract was divided into aliquots, flash-frozen and stored at –80°C.

Tagetitoxin uptake in intact and excised barley seedlings

Barley seeds were germinated and grown in a light-tight growth chamber for 4.0 days as described above. Under a green safe light, some seedlings were submerged in distilled water and excised at root level. Five excised seedlings were transferred to a vial of either distilled water or a solution of 180 μ M tagetitoxin, while five intact seedlings were placed in either distilled water or a solution of 100 μ M tagetitoxin. Seedlings were then returned to the light-tight growth chamber for 12 h. A small fan was used to blow air over the seedlings to stimulate transpiration. After the 12 h pretreatment, seedlings were transferred to the light, while others were kept in the dark, for an additional 4 h. Seedlings were then flash frozen in liquid nitrogen, ground to a fine powder and total nucleic acid isolated by phenol extraction. RNA was isolated as described above.

S1 nuclease, primer extension and DNA sequence analyses

DNA probes (restriction fragments or oligonucleotides) were 5'-end labeled with [α - 32 P]ATP according to Maniatis *et al.* (1982). S1 nuclease and primer extension assays were done as previously described (Mullet *et al.*, 1985). Results were analyzed on 5% (in Figures 2, 3 and 5) or 8% (in Figures 3, 4 and 6) polyacrylamide—8.3 M urea gels (Maxam and Gilbert, 1980). Dideoxy sequencing reactions were performed on double-stranded plasmid DNA using [α - 35 S]ATP and the Sequenase kit of US Biochemical Corp.

In vitro RNA synthesis

DNA fragments described in the text were cloned into the polylinker region of Bluescript plasmid vectors (Stratagene). T3 or T7 RNA polymerase promoters on either side of the polylinker region were used for unidirectional *in vitro* RNA synthesis across various insert DNAs after prior linearization at a vector restriction site 3' to the insert. To remove the DNA template, the resulting *in vitro*-generated transcripts (radioactively labeled or unlabeled) were treated with RQ1-DNase. The 0.87 kb RNA for use in plastid processing experiments was resuspended in 98% formamide containing 0.02% xylene cyanole and 0.02% bromophenol blue heated to 65°C and separated by electrophoresis on 1.5% agarose, 0.6 M formaldehyde/MOPs gel (Fourney *et al.*, 1987). Non-stained sample areas of the gel were excised after locating a sample in a parallel lane by staining separately with ethidium bromide. The RNA was isolated from excised bands by electroelution (Maniatis *et al.*, 1982). The eluted sample was then heated to 55°C, made 0.2 M with NaOAc and extracted twice with phenol and once with ether. The RNA was precipitated by addition of 2.5 vol ethanol, resuspended in H₂O and the A₂₆₀ determined.

In vitro transcription of exogenous DNA templates in the pea extract

Pea RNA polymerase activity on exogenous DNA templates was assayed in 20 mM HEPES—KOH pH 8, 10 mM MgCl₂, 60 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 5% glycerol, 50 μ M each of ATP, UTP, GTP and CTP and 0.15 μ g/ μ l plasmid DNA template. The reaction was initiated by the addition of the pea extract. After 60 min at 30°C, the reaction was stopped by the addition of an equal volume (200 μ l) of RNA extraction buffer (200 mM Tris—HCl pH 8.5, 300 mM NaCl, 20 mM EDTA, 1% SDS), immediately followed by phenol and 24:1 chloroform:isoamyl alcohol. After phenol extraction and EtOH precipitation, the resulting nucleic acids were treated with RQ1-DNase followed by phenol extraction and EtOH precipitation. The resulting RNA was then analyzed in primer extension experiments.

Processing of an in vitro-generated RNA in barley and pea plastid extracts

Processing assays were set up in 199 μ l volumes by addition of plastid extract (at time = 0) to an *in vitro*-transcription reaction mixture lacking plasmid DNA template and exogenous nucleotides. After 5 min at room temperature, 1–2 μ l of *in vitro*-generated RNA was added to a final concentration of 4.0×10^{-4} μ g/ μ l, and the reaction was transferred to 30°C. After 60 min, the reactions were stopped as above and the RNA from the extract was purified and assayed by primer extension analysis.

Plastid 'run-on' transcription

Run-on transcription was assayed in 5×10^7 lysed plastids (at a final concentration of 5×10^5 plastids/ μ l), as described by Klein and Mullet (1990). Briefly, transcription assays were initiated by addition of the plastids to a 100 μ l reaction mixture containing 0.5 mg/ml heparin, 20 mM HEPES—KOH pH 8, 10 mM MgCl₂, 25 mM potassium acetate, 10 mM DTT, 2 mM spermidine-(HCl)₃, 125 μ M CTP, 125 μ M GTP, 125 μ M ATP, 10 μ M unlabeled UTP and 400 μ Ci of [α - 32 P]UTP (NEN, specific activity 800 Ci/mmol). After 5 min, RNA extraction buffer (200 mM Tris—HCl pH 8.5, 300 mM NaCl, 20 mM EDTA, 1% SDS) was added,

immediately followed by addition of phenol:chloroform:isoamyl alcohol (24:24:1, vol:vol). Phenol extracted samples were passed over a sephadex G50-80 spun column, EtOH precipitated overnight at –80°C, and allowed to hybridize to RNA dot blot filters containing RNA probes A and *rbcl* or B and *rbcl*.

RNA dot blots

Plastid or 1 pmol of *in vitro*-generated RNA were denatured at 55°C for 30 min in a solution containing 20 mM morpholinisulfonic acid pH 7, 5 mM sodium acetate, 0.5 mM EDTA pH 8, 6.5% formaldehyde, and 50% formamide. After quenching on ice for 2 min, the RNAs were blotted onto Gene Screen Plus membranes (NEN) by vacuum filtration. Filters were allowed to dry at room temperature, then baked at 80°C for 2–4 h. Blots were prehybridized for 12–24 h at 55°C in 50% formamide, $5 \times$ SSC, 0.5% SDS, $2 \times$ Denhardt's solution and 100 μ g/ml denatured salmon sperm DNA [see Maniatis *et al.* (1982) for SSC and Denhardt's solutions]. Prehybridization solution was replaced by identical (fresh) hybridization solution plus α - 32 P-labeled run-on RNA or *in vitro*-generated RNA probe. After 48 h at 55°C, filters were washed at 55°C in $2 \times$ SSC, 0.5% SDS (2×5 min), then in $0.2 \times$ SSC, 0.5% SDS (2×30 min). Hybridization to duplicate dots was quantitated by liquid scintillation counting by using Betascope blot analyzer (Betagen, Framingham, MA) (Sullivan *et al.*, 1987). Under these conditions $33.3 \pm 2.8\%$ (A), $39.1 \pm 6.4\%$ (B) and $18.1 \pm 1.5\%$ (*rbcl*) of radioactively labeled *in vitro* RNA probes from regions A, B and *rbcl* hybridized to an excess of filter-bound complementary *in vitro* RNAs. *In vivo* plastid RNA levels (Table I) were quantitated by hybridization of excess labeled RNA probes to a dilution series of plastid RNA within the linear response range for each probe.

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